Materials and Methods

Mice

C57BL/6, BALB/c and IQI mice kept under SPF or GF conditions were purchased from Sankyo laboratories Japan, SLC Japan, CLEA Japan, or Jackson Laboratories USA. GF and gnotobiotic mice were bred and maintained in vinyl isolators within the gnotobiotic facility of the University of Tokyo, the Yakult Central Institute or Sankyo laboratories. *Myd88*$^{-/-}$, *Rip2*$^{-/-}$ and *Card9*$^{-/-}$ mice were generated as previously described (*S1-3*), and backcrossed for eight or more generations onto C57BL/6. Foxp3^{eGFP} mice were purchased from the Jackson Laboratories.

Generation of *Il10*^{Venus} mice

An IRES-Venus (*S4*) cassette followed by an SV40 polyadenylation signal and a neomycin resistance gene was inserted immediately before the polyadenylation signal of the *Il10* gene to generate a bicistronic locus encoding both *Il10* and Venus under the control of the *Il10* promoter. The *Il10*^{Venus} mice were re-derived into GF conditions at the Central Institute for Experimental Animals (Kawasaki, Japan) and kept in vinyl isolators at Sankyo Laboratory Services Corporation (Tokyo, Japan).

Bacteria

Mice associated with SFB or 46 strains of *Clostridium* were generated as previously described (*S5, 6*). Cecal contents or feces from these gnotobiotic mice were dissolved in sterile water and orally inoculated into GF mice. Three strains of *Lactobacillus* (*L. acidophilus*, *L. fermentum*, and *L. murinum*) (*S7*) and 16 strains of *Bacteroides* (six strains of *B. vulgatus*, seven of *B. acidifaciens* group 1, and three of *B. acidifaciens* group 2) (*S8*) were anaerobically cultured on BL and EG agar plates, respectively. Cultured bacteria were harvested, suspended in the anaerobic TS broth, and gavaged into GF mice. Fecal pellets from inoculated mice were assessed for colonization by microscopic examination of smears or qPCR for 16S rDNA.

Isolation of lymphocytes and flow cytometry

To isolate LP lymphocytes, small and large intestines were collected and opened longitudinally, washed to remove fecal content, and shaken in HBSS containing 5 mM EDTA for 20 min at 37°C. After removing epithelial cells and fat tissue, the intestines were cut into small pieces and incubated with RPMI 1640 containing 4% fetal bovine serum (FBS), 1 mg/ml collagenase D, 0.5 mg/ml dispase and 40 μg/ml DNase I (all Roche Diagnostics) for 1 h at 37°C in a shaking water bath. The digested tissues were washed with HBSS containing 5 mM EDTA, resuspended in 5 ml of 40% Percoll (GE Healthcare) and overlaid on 2.5 ml of 80% Percoll in a 15-ml Falcon tube. Percoll gradient separation was performed by centrifugation at 780 *g* for 20 min at 25°C. The interface cells were collected and used as LP lymphocytes. The collected cells were then suspended in staining buffer containing PBS, 2% FBS, 2 mM EDTA and 0.09% NaN_3 and stained for surface CD4. Intracellular staining of Foxp3 and Helios was performed using the Foxp3 Staining Buffer Set (eBioscience), because this kit permits very efficient staining of

nuclear proteins. However, because permeabilization using the eBioscience kit is often accompanied by the total loss of cytosolic Venus protein, the BD Cytofix/Cytoperm was used for concomitant detection of Venus and Foxp3, Helios and/or CTLA4 (although staining with the BD Cytofix/Cytoperm had reduced efficacy for detection of Foxp3 and Helios). For detection of IFN-γ and IL-17, lymphocytes were stimulated for 4 h with 50 ng/mL PMA (Sigma) and 1 μg/mL ionomycin (Sigma) in the presence of GolgiStop (BD Biosciences). Cells were first stained for surface CD4, then fixed and permeabilized using a BD Cytofix/Cytoperm kit, and finally stained for intracellular IFN-γ and IL-17. The following antibodies were used: PE- or PE-Cy7-labeled anti-CD4 Ab (RM4-5, BD Biosciences), Alexa647-labeled anti-Foxp3 Ab (FJK-16s, eBioscience), PE-labeled anti-Helios antibody (22F6, BioLegend), PE-labeled anti-CTLA4 antibody (UC10-4F10-11, BD Biosciences), FITC-labeled anti-IFN-γ (XMG-1.2, BD Biosciences), and PE-labeled anti-IL-17 (TC11-18H10, BD Biosciences) were used. Flow cytometry was performed using FACSCalibur or LSRII and data were analyzed with FlowJo software (TreeStar Inc.). Cell sorting was performed using a FACSAria with a resulting purity of around 95%.

Preparation and culture of colonic IECs

Colons were isolated, opened longitudinally and rinsed with PBS. The epithelial integrity was disrupted by treatment with 1 mM dithiothreitol (DTT) for 30 min at 37°C on a shaker, followed by vortexing for 1 min. The liberated IECs were collected, resuspended in 5 ml of 20% Percoll and overlaid on 2.5 ml of 40% Percoll in a 15-ml Falcon tube. Percoll gradient separation was performed by centrifugation at 780 *g* for 20 min at 25°C. The interface cells were collected and used as colonic IECs (purity >90%; survival rate 95%). IECs were then suspended in RPMI 1640 containing 10% FBS and cultured in 24 well plates at 1×10^5 cells for 24 hours. Culture supernatants were collected and the level of active-form of TGF-β1 was measured by ELISA (Promega). For *in vitro* T cell culture, MACS-purified splenic $CD4^+$ T cells were cultured in 96-well round bottom plates at 1.5×10^5 cells/well with 50% conditioned medium from cultures of IECs obtained from GF or *Clostridium*-colonized mice, 25 ng/ml hIL-2 (Peprotech), 10 μg/ml plate-bound anti-CD3 and anti-CD28 (BD Bioscience) in the presence or absence of 25 μg/ml anti-TGF-β Ab (R&D). After 5 days of incubation, $CD4^+$ T cells were collected and real-time RT-PCR was performed.

In vitro suppression assay

$CD4^+CD25^-$ cells (Teff cells) were sorted from spleens of SPF C57BL/6 mice and plated at 2×10^4/well in 96-well round-bottomed plates with 2×10^4 irradiated (30 Gy) splenic $CD11c^+$ cells, 0.5 μg/ml anti-CD3 antibody, and various numbers of $CD4^+Venus^+$ cells from colonic lamina propria of SPF or *Clostridium*-associated *Il10*^{Venus} mice for 3 days. [^{3}H]-thymidine (1 μCi/well) was added during the last 6 hours of culture.

Experimental colitis model

C57BL/6 mice were inoculated with fecal suspensions from *Clostridium*-associated mice at 2 weeks old and reared under conventional conditions for 6 weeks. For DSS-induced colitis, mice were given 2% (wt/vol) DSS (reagent-grade DSS salt; molecular mass = 36–50 kD; MP Biomedicals) in drinking water for 6 days. For oxazolone-induced colitis, mice were pre-sensitized by epicutaneous application of 3% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma-Aldrich) in 100% ethanol in a volume of 150 μl. Five days after pre-sensitization mice were re-challenged intra-rectally with 150 μl 1% oxazolone in 50% ethanol under light anesthesia using a 3.5 F catheter. Body weight, hemoccult, gross blood, and stool consistency were analyzed on a daily basis. The disease score was calculated by scoring percent weight loss, intestinal bleeding [no blood, occult blood (hemoccult +), or gross blood], and stool consistency (normal stool, loose stool, or diarrhea), as previously described (*S9*).

OVA-specific IgE responses

BALB/c SPF mice were inoculated with the fecal suspension of *Clostridium*-associated mice at 2 weeks old and reared under conventional conditions. Mice were injected intraperitoneally with 1 μg of OVA (Grade V, Sigma) and 2 mg of Alum (Thermo Scientific) in a total volume of 0.2 ml at 4 and 6 weeks of age. Serum was collected from the base of the tail every week, and OVA-specific IgE was measured by ELISA (Chondrex). Splenocytes were collected at 8 weeks of age, plated at 1 x 10^6/well in 96-well plates and stimulated with OVA (100 μg/ml) for 3 days. Culture supernatants were collected and IL-4 and IL-10 levels were measured by ELISA (R&D).

16S rRNA gene quantitative PCR analysis

Bacterial genomic DNA was isolated from fecal pellets or luminal contents using a QIAamp DNA Stool mini kit (QIAGEN). Quantitative PCR analysis was carried out using a LightCycler 480 (Roche). Relative quantity was calculated by the ΔCt method and normalized to the amount of total bacteria, dilution, and weight of the sample. The following primer sets were used: total bacteria, 5'-GGTGAATACGTTCCCGG-3' and 5'-TACGGCTACCTTGTTACGACTT-3' (*S10*); *Enterobacteriaceae*, 5'-TGCCGTAACTTCGGGAGAAGGCA-3'and 5'-TCAAGGACCAGTGTTCAGTGTC-3' (*S11*); *Clostridium leptum*, 5'-CCTTCCGTGCCGSAGTTA-3' and 5'-GAATTA AACCACATACTCCACTGCTT-3' (*S12*); *Clostridium coccoides*, 5'-AAATGACGGTACCTGACTAA-3' and 5'-CTTTGAGTTTCATTCTTGCGAA-3' (*S13*); *Lactobacillus fermentum*, 5'-CCTGATTGATTTTGGTCGCCAAC-3' and 5'-ACGTATGAACAGTTACTCTCATACGT-3' (*S14*); *Clostridium* spp. (strain9), 5'-CACCAAGGCGACGATCAGT-3' and 5'-GAGTTTGGGCCGTGTCTCA-3'; (strain41), 5'-GCAAGTCGAACGGAGATATCATT-3' and 5'-CTCACCCGTCCGCCACTA-3'; (strain27), 5'-GAAACAGCTGCTAATACCGCATAA-3' and 5'-TCAGACGCGAGTCCATCTCA-3'; (strain5), 5'-CACGTGAGCAACCTGCCTTT-3' and 5'-TGCCATGCGACTCCGATA-3'; (strain18), 5'-GGATGGACGAGAGATAAGCTTAGTG-3' and 5'-CCTCTGAAAGGCAGGTTGCT-3'; (strain3), 5'-AGATGGCCTCGCGTCTGAT-3' and 5'-GCCGGCCAACCTCTCAGT-3'.

Real-time RT-PCR

RNA samples were prepared using an RNeasy Mini Kit (Qiagen) and complementary DNAs were synthesized using MMV reverse transcriptase (Promega). Real-time RT-PCR was performed using SYBR Premix Ex Taq (TAKARA) and a LightCycler 480. Values were then normalized to the expression of GAPDH for each sample. The primer sets were designed with Primer Express Version 3.0 (Applied Biosystems) and were initially tested to confirm comparable (> 90%) efficiencies. The following primer sets were used: Foxp3, 5'-GGCAATAGTTCCTTCCCAGAGTT-3' and 5'-GGGTCGCATATTGTGGTACTTG-3'; Mmp2, 5'-GGACATTGTCTTTGATGGCA-3' and 5'- CTTGTCACGTGGTGTCACTG-3'; Mmp9, 5'-TCTCTGGACGTCAAATGTGG-3' and 5'- GCTGAACAGCAGAGCCTTC-3'; Mmp13, 5'-AGGTCTGGATCACTCCAAGG-3' and 5'- TCGCCTGGACCATAAAGAA-3'; Ido1, 5'-AGAGGATGCGTGACTTTGTG-3' and 5'- ATACAGCAGACCTTCTGGCA-3'; IL-10, 5'-GATTTTAATAAGCTCCAAGACCAAGGT-3' and 5'-CTTCTATGCAGTTGATGAAGATGTCAA-3'; GAPDH, 5'-CCTCGTCCCGTAGACAAAATG-3' and 5'-TCTCCACTTTGCCACTGCAA-3'.

Statistical analysis

Differences between control and experimental groups were evaluated using Unpaired-Student's *t*-test.

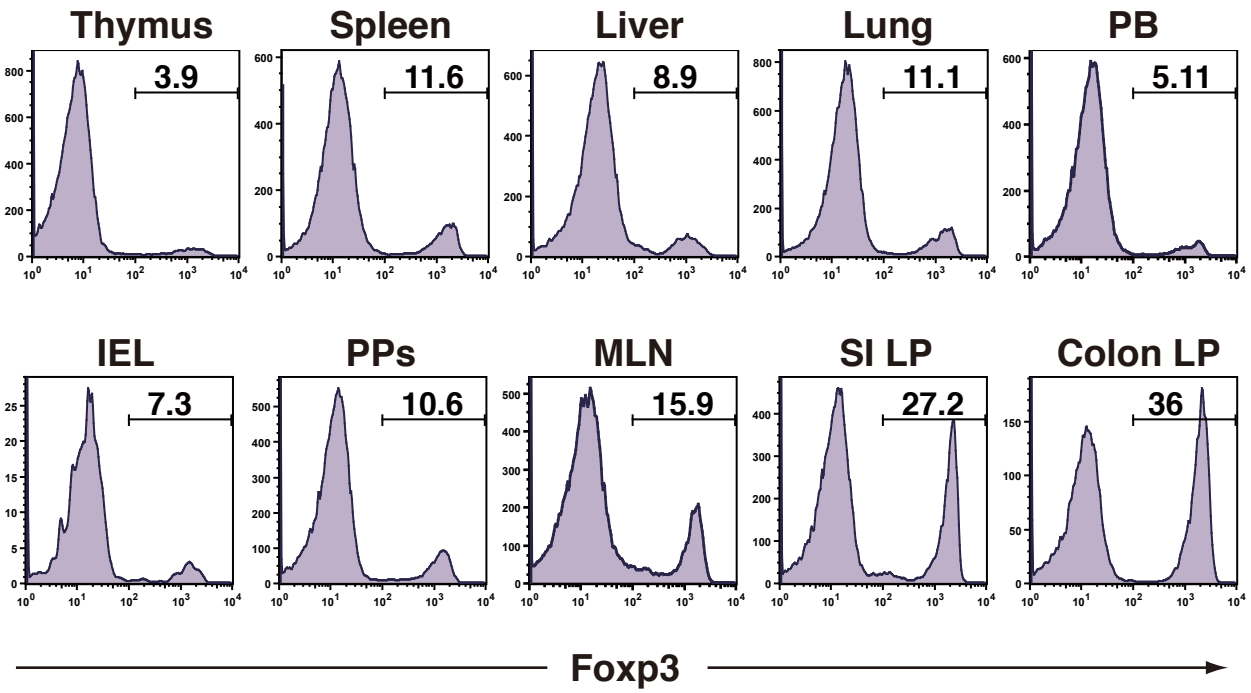

fig. S1. Foxp3-positive cells are most abundant at intestinal mucosa.
Lymphocytes from thymus, spleen, liver, lung, peripheral blood (PB), intraepithelial lymphocytes (IELs), Peyer's patches (PPs), mesenteric lymph nodes (MLN), or the lamina propria (LP) of small intestine (SI) or colon of specific-pathogen-free (SPF) Foxp3-GFP mice were analyzed for CD4 and GFP (Foxp3) by flow cytometry. Representative histograms gated on $CD4^+$ cells from one of three independent experiments are shown. Numbers indicate the percentages of cells in the gate.

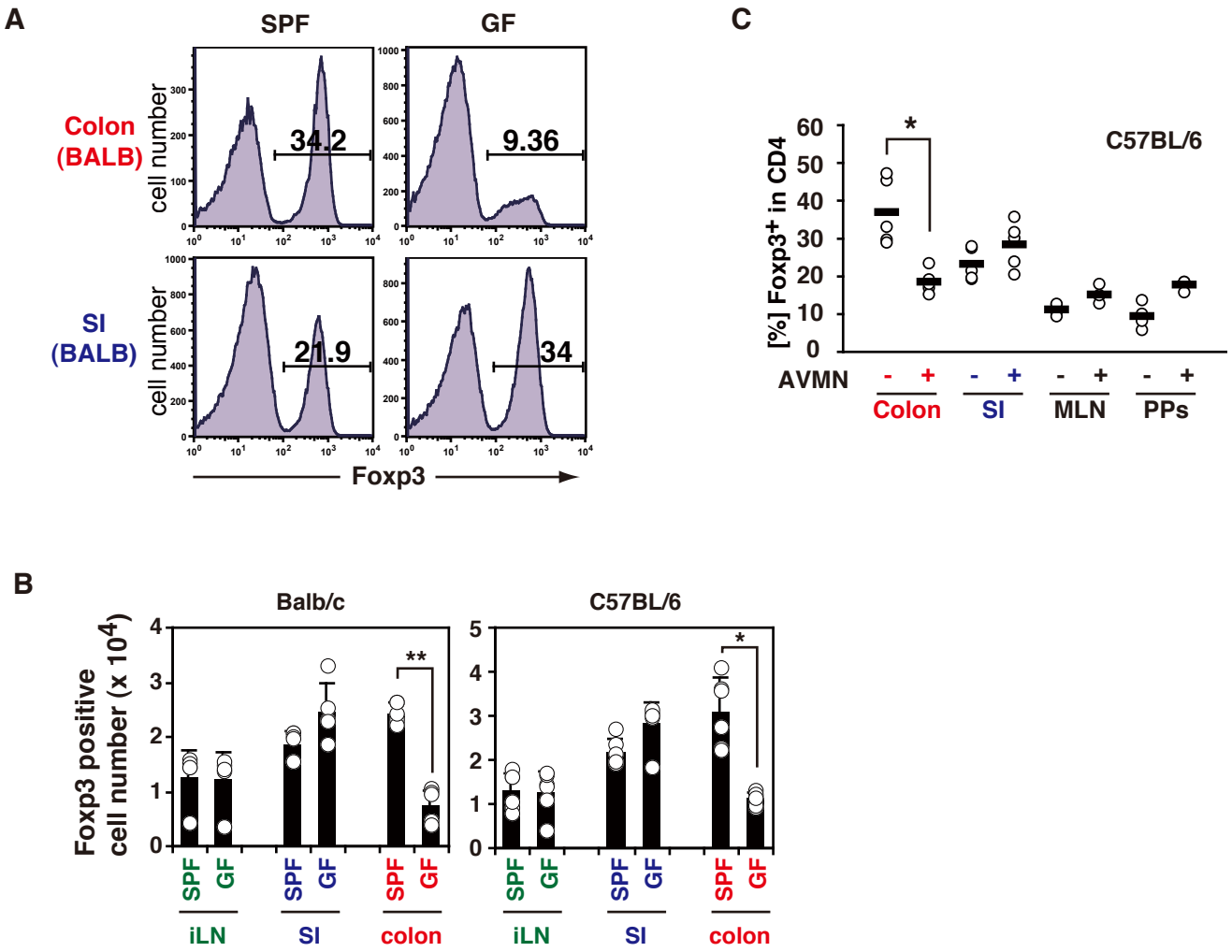

fig. S2

(A, B) The percentage and absolute number of Foxp3$^+$CD4$^+$ T cells in iLN, SI and colon of SPF and GF mice. Lymphocytes were isolated from inguinal lymph nodes (iLN) or the lamina propria (LP) of colon or small intestine (SI) of SPF BALB/c mice and C57BL/6 mice under SPF and germ-free (GF) conditions, and stained for CD4 and Foxp3. Representative histograms gated on CD4$^+$ cells are shown in (A). The absolute number of CD4$^+$Foxp3$^+$ cells in individual mice is shown in (B). Data are representative of three independent experiments.

(C) Reduction of Foxp3$^+$ Treg cells in colonic LP of SPF mice treated with antibiotics. SPF C57BL/6 mice were given a combination of antibiotics [ampicillin (A; 500 mg/L; Sigma), vancomycin (V; 500 mg/L; Nacalai Tesque), neomycin (N; 1 g/L; Nacalai Tesque) and metronidazole (M; 1 g/L; Nacalai Tesque)] (refs. *S15,16*) in the drinking water for 8 weeks (4 or 5 mice per group). Lymphocytes in the LP of colon and SI, mesenteric LN, and Peyer's patches (PPs) were stained for CD4 and Foxp3, and analyzed by flow cytometry. The percentage of Foxp3$^+$ cells within the CD4$^+$ cell population in individual mice is shown. Horizontal bars indicate the mean. Data are from two independent experiments with similar results.

Each circle in (B,C) represents a separate animal, and error bars indicate the SD (n ≥5 mice per group). $^{**}P < 0.001$; $^{*}P < 0.01$, unpaired *t*-test.

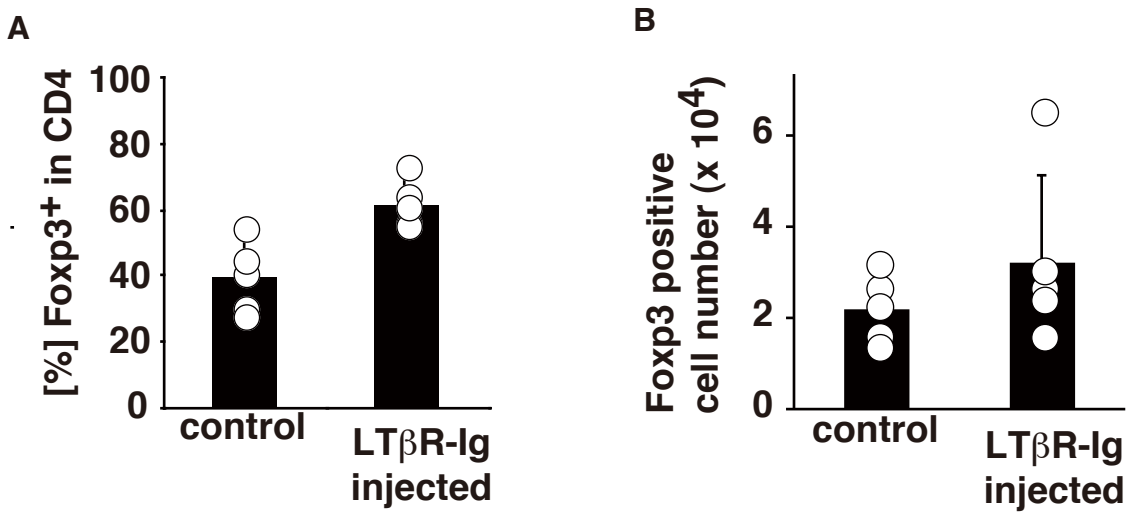

fig. S3 Lamina propria Treg cells in ILF-, PP- and colonic-patche-null mice.
Pregnant SPF C57BL/6 mice were injected i.p. with 100 μg of recombinant protein of the extracellular domain of lymphotoxin β receptor (LTβR) fused to the Fc portion of human IgG1 (LTβR-Ig) on gestational day 14. The progeny were again i.p. injected with LTβR-Ig to completely remove the isolated lymphoid follicles (ILF), Peyer's patches (PP) and colon patches (CP) (refs. *S17-23*). The percentage (**A**) and absolute number (**B**) of $Foxp3^+$ cells within the lamina propria $CD4^+$ cell population in the LTβR-Ig- or control rat IgG-treated mice was evaluated by flow cytometry. Each circle represents individual mice and error bars represent the SD (n =5 mice per group). Data are representative of two independent experiments.

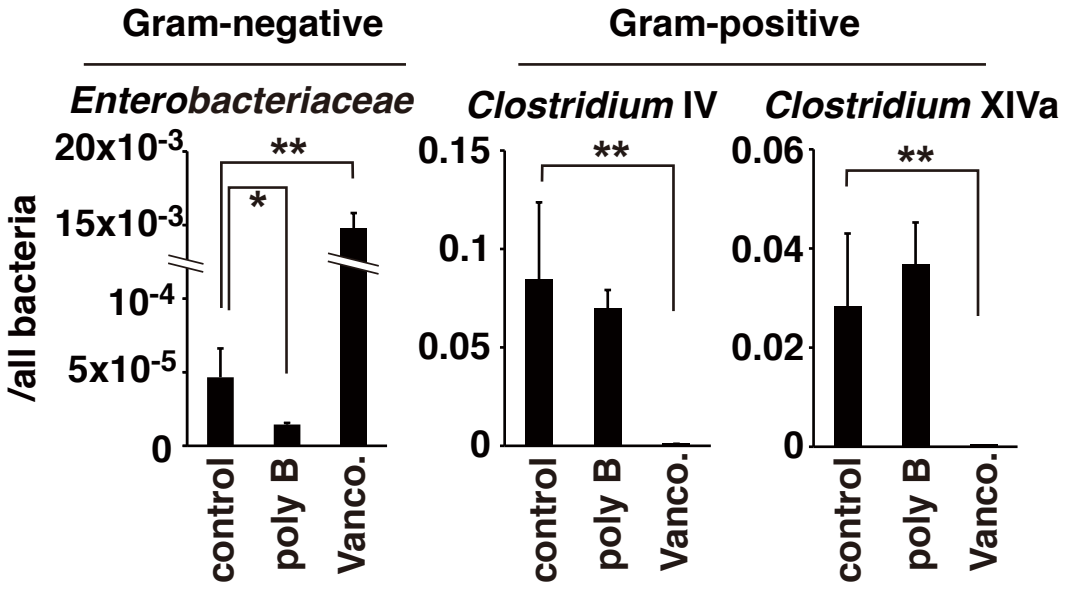

fig. S4 Changes in the amounts of Gram-negative and -positive bacteria in feces of mice treated wihth polymyxin B or Vancomycin.

Four-week old SPF C57BL/6 mice were given polymyxin B (poly B: 100 mg/l) or vancomycin (Vanco: 500 mg/l) in the drinking water for 4 weeks. Bacterial DNA was extracted from feces using the QIAamp DNA Stool mini kit (QIAGEN). Quantitative PCR analysis for 16s rRNA coding DNA was carried out using a LightCycler 480 (Roche). Primer sequences for total bacteria, *Enterobacteriaceae*, *Clostridium* cluster IV (*C. leptum* group) and *Clostridium* cluster XIVa (*C. coccoides* group) were as described previously (refs. *S10*, *11*, *12* and *13*, respectively). The relative quantity of bacteria was calculated by the ΔCt method and normalized to the amount of total bacteria in each sample. Data are representative of two independent experiments and error bars represent the SD (n ≥4 mice per group). $^{**}P < 0.001$; $^{*}P < 0.01$, unpaired *t*-test.
Note that mice treated with vancomycin had significantly lower amounts of Gram-positive clostridia, accompanied by a relative increase in the amounts of Gram-negative *Enterobacteriaceae*, compared with the controls. In contrast, treatment with polymyxin B reduced Gram-negative *Enterobacteriaceae*, but did not affect Gram-positive clostridia.

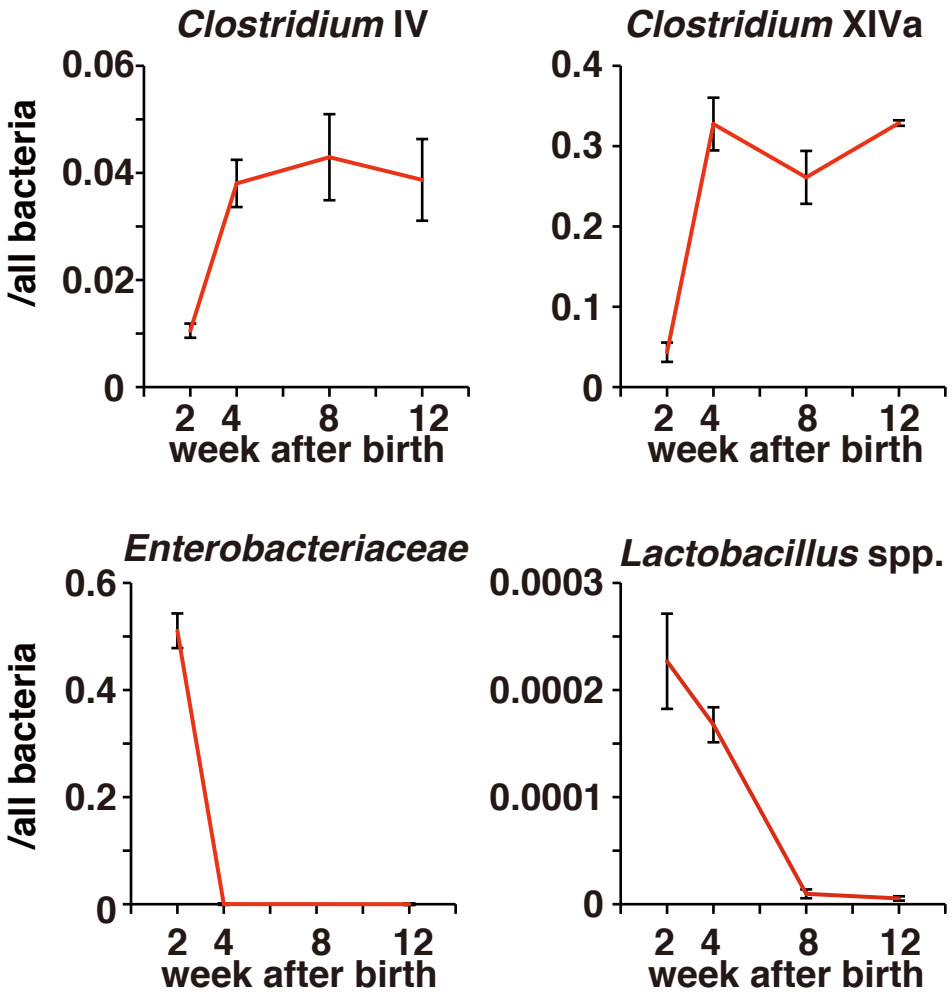

fig. S5 Appearance of clostridia during ontogeny in SPF mice.
Bacterial DNA from the feces of Balb/c SPF mice at the indicated age was extracted and analyzed by q-PCR for 16s rRNA coding gene as described in fig. S4 using primer sets for *Enterobacteriaceae*, *Clostridium* cluster IV (*C. leptum* group) and *Clostridium* cluster XIVa (*C. coccoides* group) and *Lactobacillus* spp.(*L. fermentum*). The relative quantity of bacteria was calculated by the ΔCt method and normalized to the amount of total bacteria in each sample. Error bars represent the SD. The feces from individual mice (n =3) at indicated age were analyzed. Primer sequences for *L. fermentum* were as described by ref. *S14*.
Note that the temporal change in the amount of intestinal clostridia correlated with the age-dependent accumulation of colonic Treg cells (see Fig. 1A).

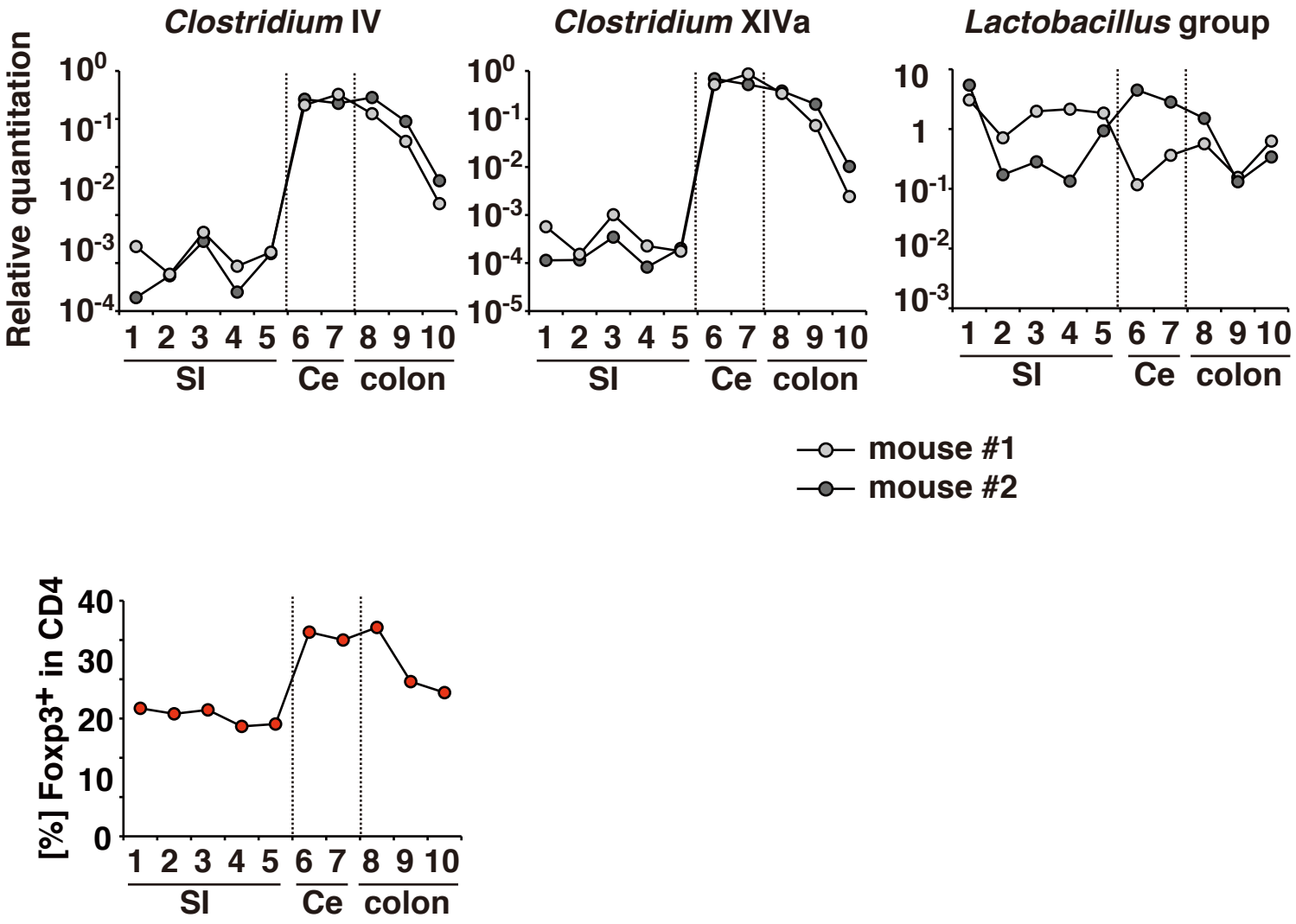

fig. S6 Anatomical distribution of *Clostridium* and Treg cells in the intestinal mucosa.
The small intestine (SI), cecum (Ce), and colon from two C57BL/6 SPF mice were subdivided into 10 segments (segment 1-5 from SI, 6-7 from Ce, and 8-10 from colon). DNA from luminal contents of each segment was extracted and analyzed for for 16S rDNA sequences of *Clostridium* IV and XIVa groups, and *Lactobacillus* spp. by q-PCR as described in figS4 and fig.S5. The relative quantity of bacteria was calculated by the ΔCt method and normalized by the dilution and weight of the sample (upper panels).
The percentage of lamina propria Foxp3^{+} cells within CD4 T cells in each segment was also analyzed (bottom panel).

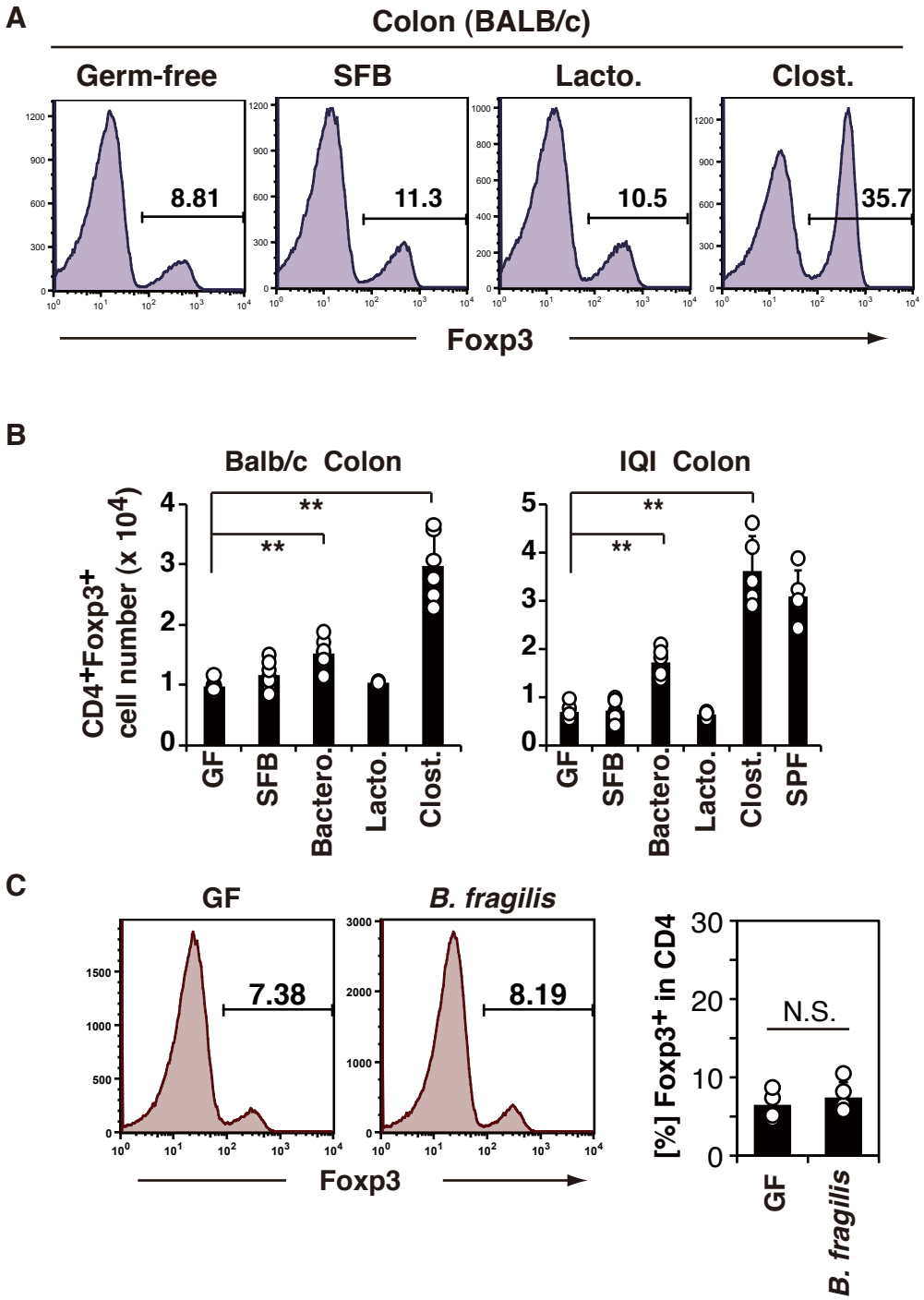

fig. S7

(**A, B**) **Foxp3$^+$ cells in colonic lamina propria of gnotobiotic mice.** Germ-free (GF) BALB/c or IQI mice were colonized with segmented filamentous bacteria (SFB), 16 strains of *Bacteroides* (Bactero.), 3 strains of *Lactobacillus* (Lacto.), or 46 strains of *Clostridium* (Clost.). The colonic lamina propria lymphocytes were collected, stained for CD4 and Foxp3, and analyzed by FACS. Representative FACS histograms gated on CD4$^+$ cells from BALB/c mice are shown in (A). The absolute number of CD4$^+$Foxp3$^+$ cells in individual mice is shown in (B).

(**C**) **Treg cells in mice colonized with *Bacteroides Fragilis*.** GF IQI mice were colonized with *Bacteroides Fragilis* (NCTC9343). Three weeks after colonization, Treg cells in the colonic lamina propria were analyzed by flow cytometry. Representative FACS histograms gated on CD4$^+$ cells are shown in the left panels. The percentage of Foxp3$^+$ cells within the CD4$^+$ cell population of individual mice is shown in the right graph.

Each circle in (B,C) represents a separate animal, and error bars indicate the SD (n ≥5 mice per group). $^{**}P <$ 0.001, N.S., statistically not significant, unpaired *t*-test. Data are representative of three independent experiments.

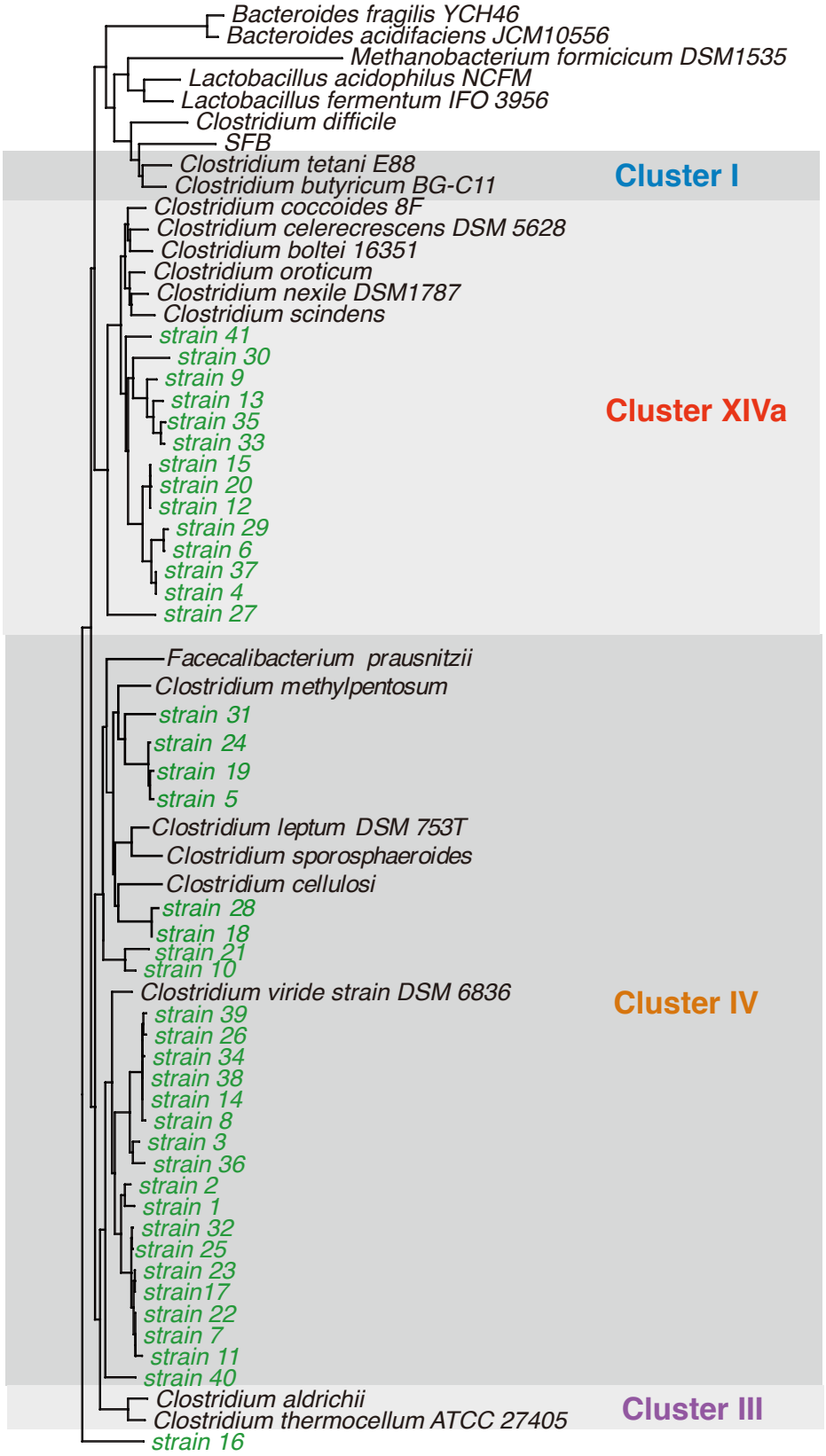

fig. S8 Phylogenetic distribution of 16S rRNA sequences of bacterial species residing in *Clostridium*-colonized mice.

DNA was extracted directly from the colonic luminal content of *Clostridium*-colonized mice. The 16S rRNA genes were amplified by PCR using 16S rRNA gene-specific primer pairs 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-ACGGCTACCTTGTTACGACTT-3' (ref. *S24*). The 1.5-kb PCR product was then introduced into pCR-Blunt Vector. The inserts were sequenced and aligned using the ClustalW software program. Phylogenetic tree was constructed by the neighbor-joining method with the resulting sequences of 41 strains of *Clostridium* (green) and those of known bacteria obtained from the GenBank database (black) using Mega software .

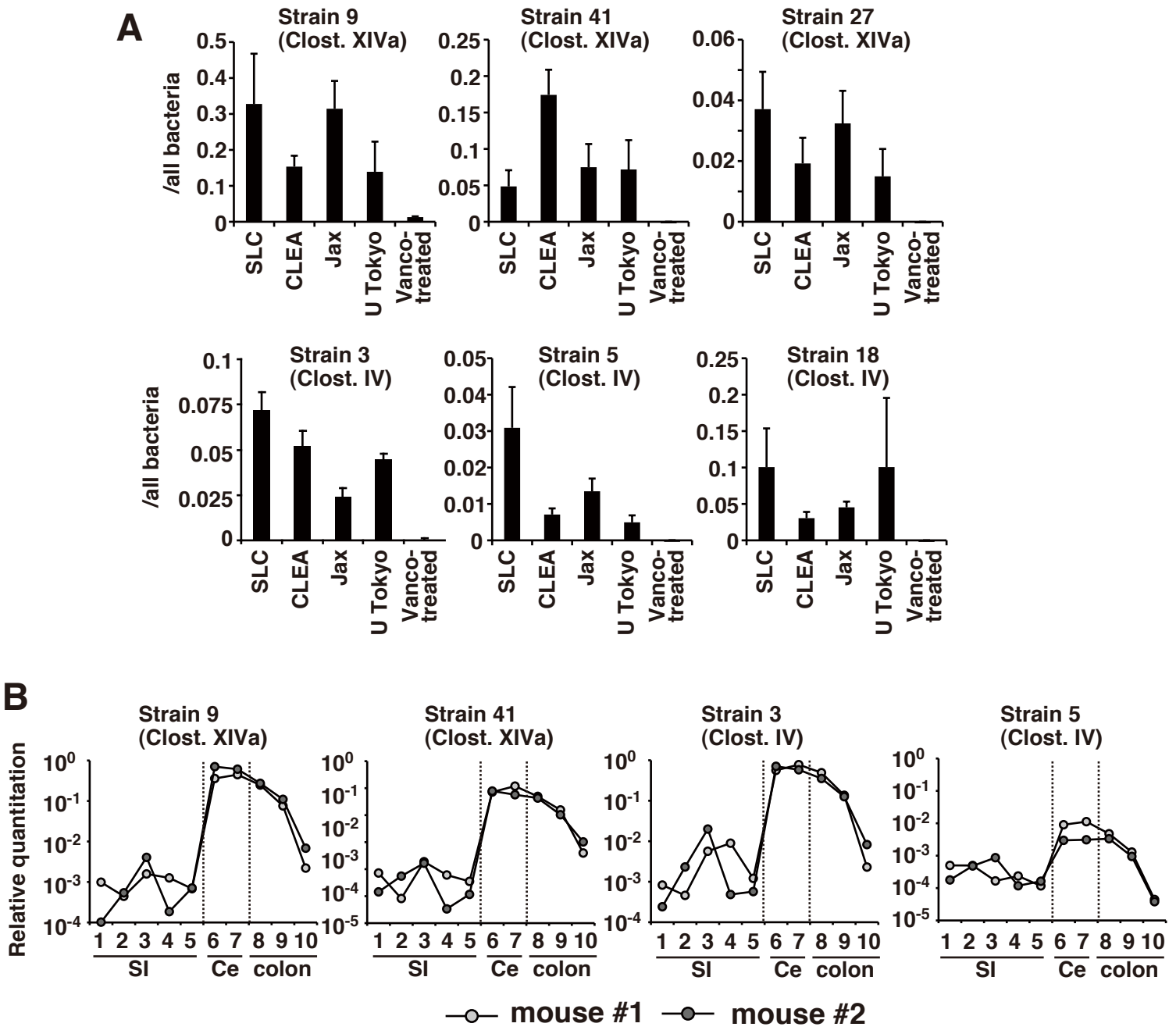

fig. S9

(**A**) **Presence of *Clostridium* strains in mice from several animal facilities.** DNA was extracted from the cecal contents or feces of SPF mice kept in SLC Japan, CLEA Japan, or Jackson Laboratories USA, and our own animal facility, and from the feces of mice treated with vancomycin for 4 weeks. The relative quantity of each *Clostridium* strain was analyzed by q-PCR as described in figS4. Error bars represent the SD (n ≥3 mice per group). Data are representative of two independent experiments.

(**B**) **Anatomical distribution of *Clostridium* strains in intestines of SPF C57BL/6 mice.** DNA from luminal contents of each segment of the intestine was extracted and analyzed by q-PCR as described in figS6. The relative quantity of bacteria was calculated by the ΔCt method and normalized by the dilution and weight of the sample. The relative quantity of bacterial cells in each section is shown.

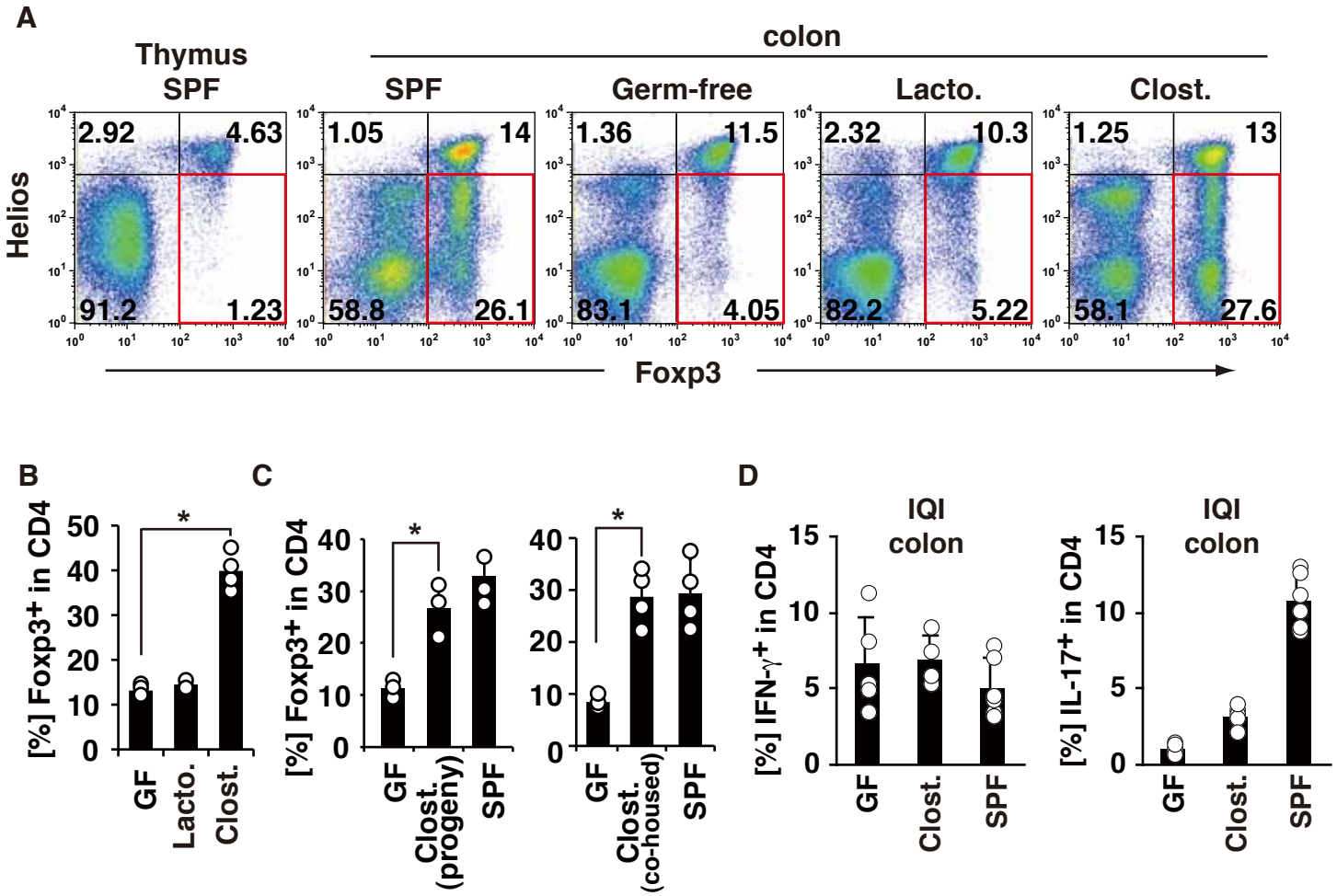

fig. S10

(**A**) **Helios expression in Foxp3$^+$ cells in the colonic LP of mice colonized with *Clostridium*.** Lymphocytes from the thymus or colonic LP of SPF, Germ-free, 3 strains of *Lactobacillus* (Lacto.)- or 46 strains of *Clostridium* (Clost.)- associated mice were analyzed for the expression of CD4, Foxp3 and Helios by flow cytometry. Representative plots for Helios and Foxp3 expression gated on CD4$^+$ cells are shown.

(**B**) **Foxp3$^+$ cells in the colonic LP of mice 4 monts after the colonized of *Clostridium*.** IQI germ-free (GF) mice were colonized with 3 strains of *Lactobacillus* (Lacto.) or 46 strains of *Clostridium* (Clost.). The colonic LP lymphocytes were collected, stained for CD4 and Foxp3, and analyzed by FACS. The percentages of Foxp3$^+$ cells within the CD4$^+$ cells in the colon of mice 4 months after the inoculation are shown.

(**C**) **Clostridium-mediated Treg induction is vertically and horizontally transmissible.** (**left**) The progeny mice from Clost-colonized mice were reared with Clost-colonized mothers in a vinyl isolator for 8 weeks. The percentages of Foxp3$^+$ cells within colonic CD4$^+$ cells in the adult progeny mice were analyzed by FACS. (**right**) GF IQI mice (8-week old) were cohoused with Clost-colonized mice for 3 weeks before analyzing the percentages of Foxp3$^+$ cells within colonic CD4$^+$ cells.

(**D**)**Th1 and Th17 cells in the colonic LP of mice colonized with *Clostridium*.** IQI GF mice were colonized with *Clostridium* (Clost.) or microbiota from SPF mice (SPF). Colonic LP lymphocytes were isolated 3 weeks later and the percentage of IFN-γ$^+$ cells or IL-17$^+$ cells within the CD4$^+$ lymphocyte population was analyzed by FACS. Note that the colonization by *Clostridium* had no effect on the induction of Th1 cells and a moderate effect on the induction of Th17 cells.

Data are representative of two independent experiments. Each circle represents a separate animal (B-D). Error bars indicate the SD (n ≥3 mice per group) (B-D). *$P < 0.02$, unpaired *t*-test.

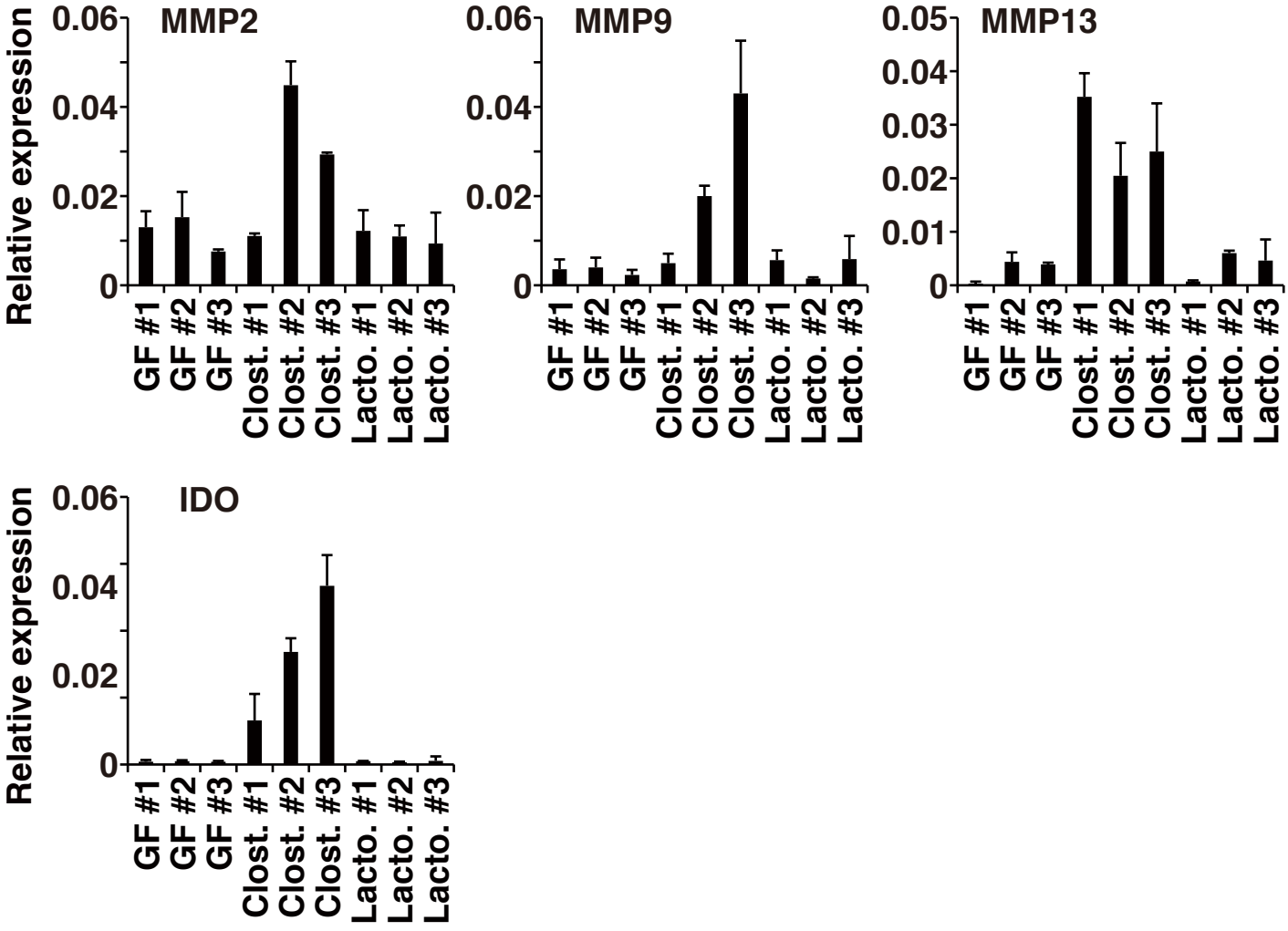

Fig. S11. Expression of matrix metalloproteinases (MMPs) and indoleamine 2,3-dioxygenase (IDO) in colonic IECs of *Clostridium*-colonized mice.

C57BL/6 germ-free (GF) mice were orally inoculated with 46 strains of *Clostridium* (Clost.) or 3 strains of *Lactobacillus* (Lacto.). Ten days after inoculation, epithelial cells (IECs) were collected and the relative mRNA expression levels of MMP2, 9, 13 or IDO genes were analyzed by real-time RT-PCR. Each group consisted of three mice, and error bars indicate the SD.

MMP2, MMP9, and MMP13 were proposed to activate latent TGF-β (refs. *S25-27*).

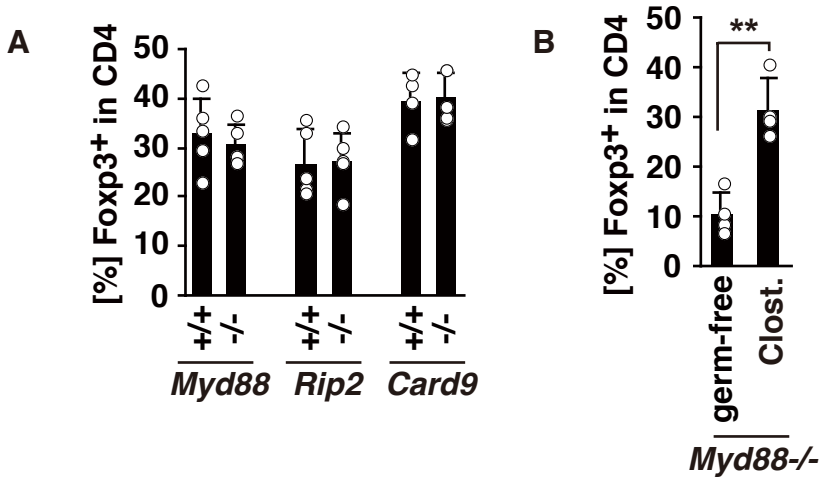

Fig. S12. Role of bacterial pattern-recognition receptors (PRRs) to the induction of Treg cells by *Clostridium*.

(**A**) Lymphocytes were isolated from the colonic lamina propria of SPF Myd88-/-, Rip2-/- or Card9-/- mice, and stained for CD4 and Foxp3. The percentage of $Foxp3^+$ cells within the $CD4^+$ cell population in the colon were shown.

(**B**). Germ-free Myd88-/- mice were colonized with 46 strains of *Clostridium* (Clost.) and the percentage of $Foxp3^+$ cells within the $CD4^+$ cell population was analyzed 3 weeks later. The percentage of $Foxp3^+$ cells within the $CD4^+$ cell population in the colon were shown.

Each circles represents a separate animal, and error bars indicate the SD (n =5 mice per group). $^{**}P < 0.001$, unpaired *t*-test. Data are representative of two independent experiments.

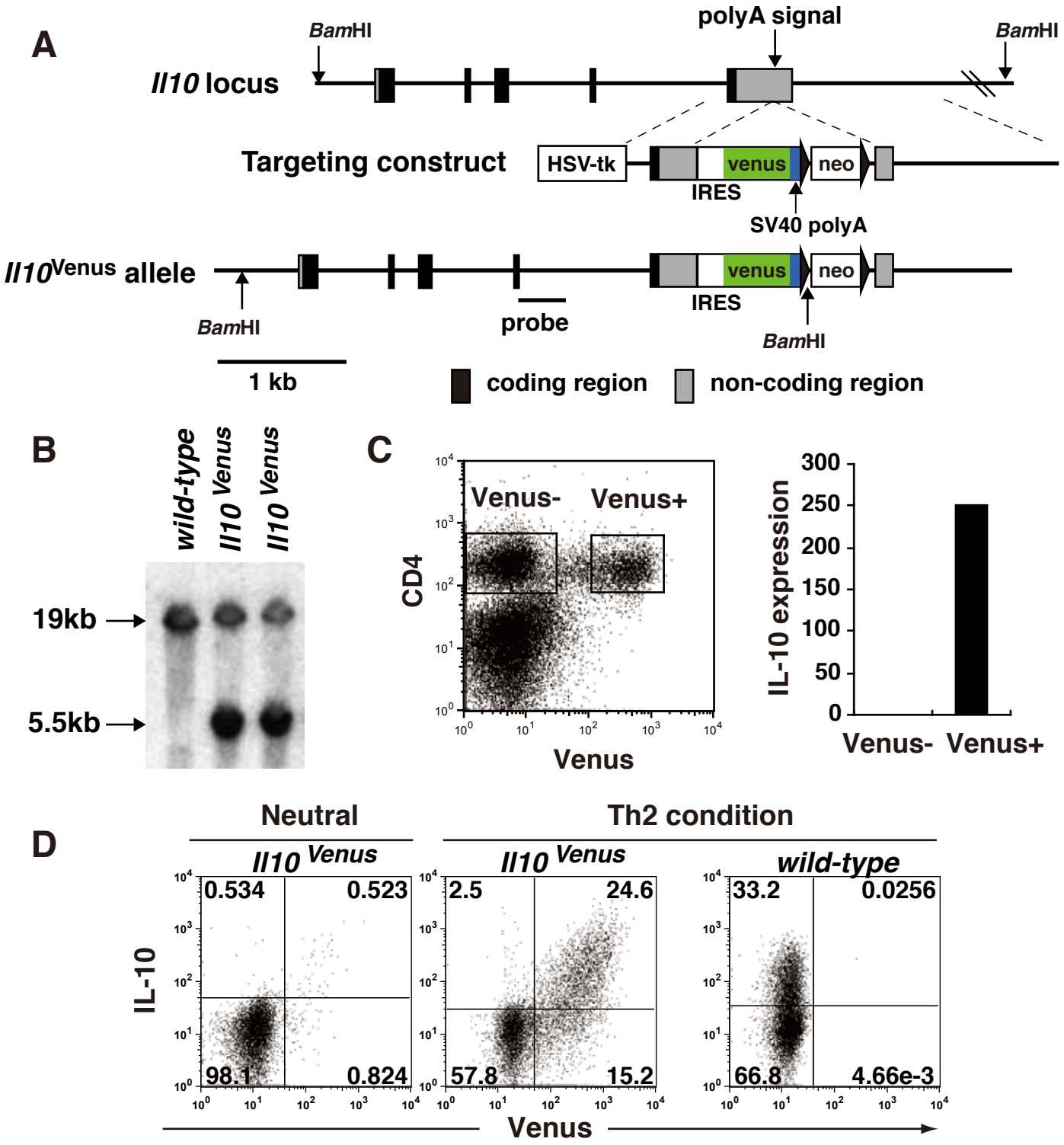

fig. S13 Generation of IL10^{Venus} mice.

(**A**) An IRES-Venus-SV40 polyA cassette followed by the neomycin-resistance gene was inserted immediately before the polyadenylation signal of exon 5 of the *Il10* gene to obtain the targeting construct. Homologous recombination resulted in the final IL10^{Venus} allele (bottom). tk, thymidine kinase; neo, neomycin-resistance gene.

(**B**) Genomic DNA was purified from mice, digested with BamHI, and Southern blotting was performed using the probe shown in (A). Wild-type and IL10^{Venus} alleles were detected as 19-kb and 5.5-kb bands, respectively.

(**C**) Colonic lamina propria CD4^{+}Venus^{-} or CD4^{+}Venus^{+} cells were sorted from IL10^{Venus} mice using a FACSAria and examined for the expression of IL-10 mRNA by real-time RT-PCR using an ABI 7300 system. The expression of IL-10 mRNA was only detected in the CD4^{+}Venus^{+} cells.

(**D**) Naive CD4^{+} T cells from spleen of IL10^{Venus} mice or WT mice were cultured with splenic CD11c^{+} cells and anti-CD3 under neutral conditions or Th2 polarizing conditions (1000 U/ml of mouse recombinant IL-4 and 10 μg/ml of anti-IFN-γ (XMG1.2)) for 4 days. After stimulation with PMA and ionomycin, intracellular cytokine staining was performed using anti IL-10-APC (JES5-16E3, BD Biosciences). Representative flow cytometry plots gated on CD4^{+} cells are shown.

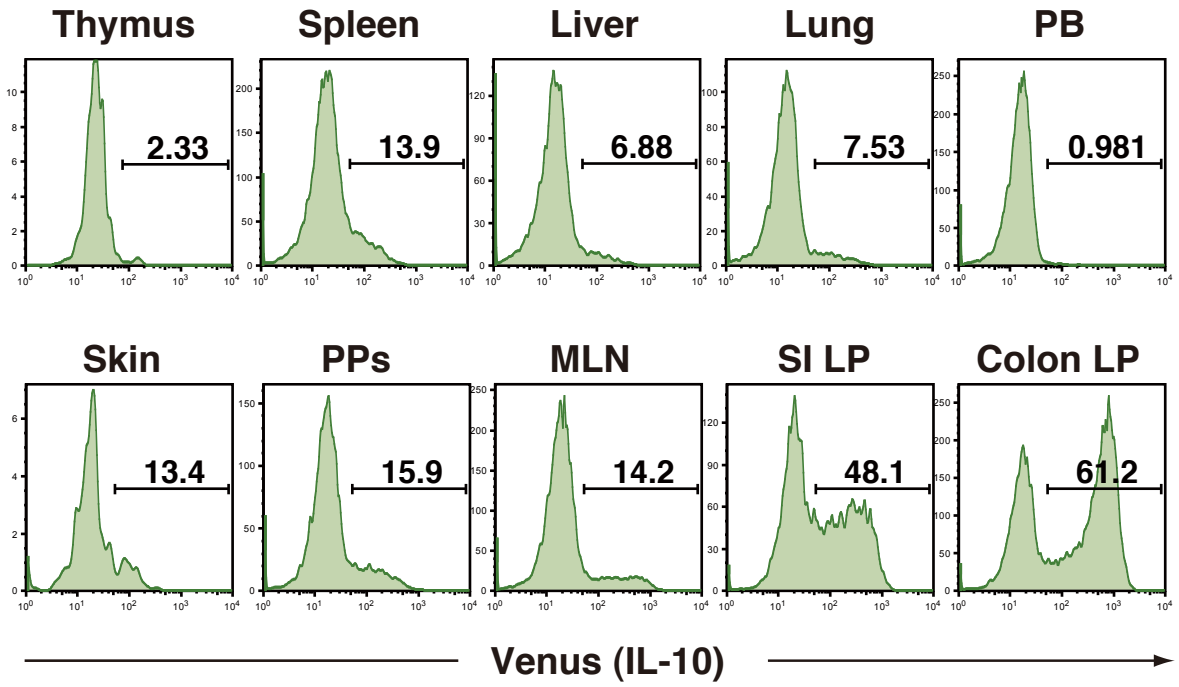

fig. S14. IL-10 producing Foxp3-positive cells in various organs.
Lymphocytes from thymus, spleen, liver, lung, peripheral blood (PB), skin, Peyer's patches (PPs), mesenteric lymph nodes (MLN), or the lamina propria (LP) of small intestine (SI) or colon of SPF IL10^{Venus} mice were analyzed for CD4, Foxp3 and Venus (IL-10) by flow cytometry. Representative histograms gated on CD4^{+} Foxp3^{+} cells from one of three independent experiments are shown. Numbers indicate the percentages of cells in the gate.

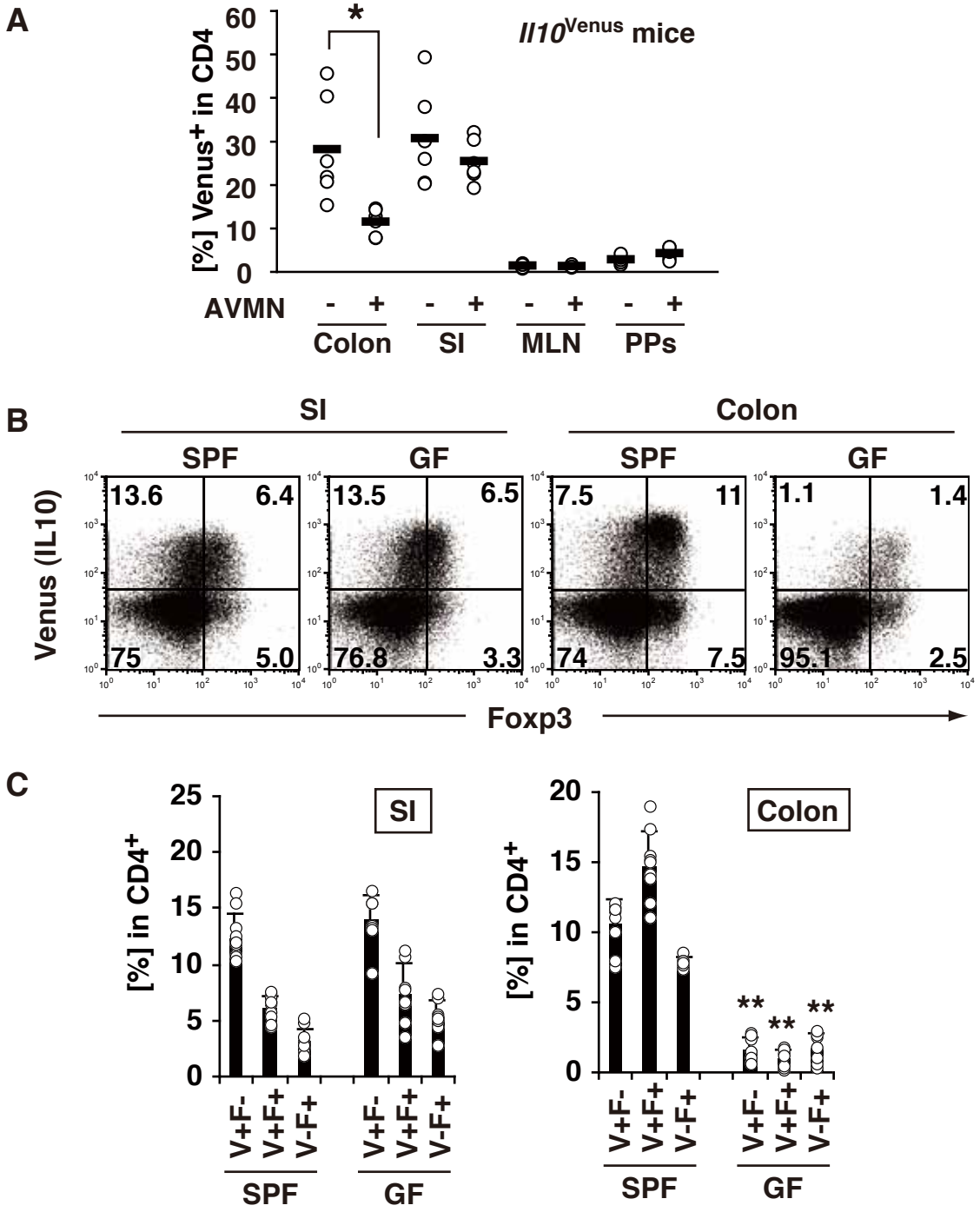

fig. S15

(**A**) **Venus$^+$ cells in the colonic lamina propria of mice treated with antibiotics.** IL10^{Venus} SPF mice were given a combination of antibiotics [ampicillin (A; 500 mg/L), vancomycin (V; 500 mg/L), neomycin (N; 1 g/L) and metronidazole (M; 1 g/L)] in the drinking water for 10 weeks (5 or 6 mice per group). Lymphocytes in the colon LP, SI LP, MLNs, and PPs of the mice were stained for CD4, and analyzed by FACS. The percentage of Venus$^+$ cells within CD4$^+$ cells of individual mice is shown. Horizontal bars indicate means. Data are from two independent experiments with similar results (n ≥5 mice per group). *$P < 0.02$, unpaired *t*-test.

(**B, C**) **Foxp3 and IL-10 expression in intestinal CD4$^+$ cells of SPF and GF mice.** The colonic and SI LP lymphocytes from IL10^{Venus} mice kept under germ-free (GF) or SPF conditions were analyzed for the expression of CD4, Foxp3 and Venus (IL-10) by flow cytometry. Representative plots for Foxp3 and Venus expression by CD4$^+$ cells are shown in (B). The numbers indicate the percentage of cells in each quadrant. The percentage of Foxp3- and/or Venus-expressing subsets (V+ F-, Venus$^+$ Foxp3$^-$ cells; V+ F+, Venus$^+$Foxp3$^+$ cells; V-F+, Venus$^-$Foxp3$^+$ cells) within the CD4$^+$ cell population in individual mice is shown in (C). Error bars represent the SD. Data are representative of three independent experiments (n ≥6 mice per group). **$P < 0.001$ vs. SPF, unpaired *t*-test.

Each circle in (A,C) represents a separate animal.

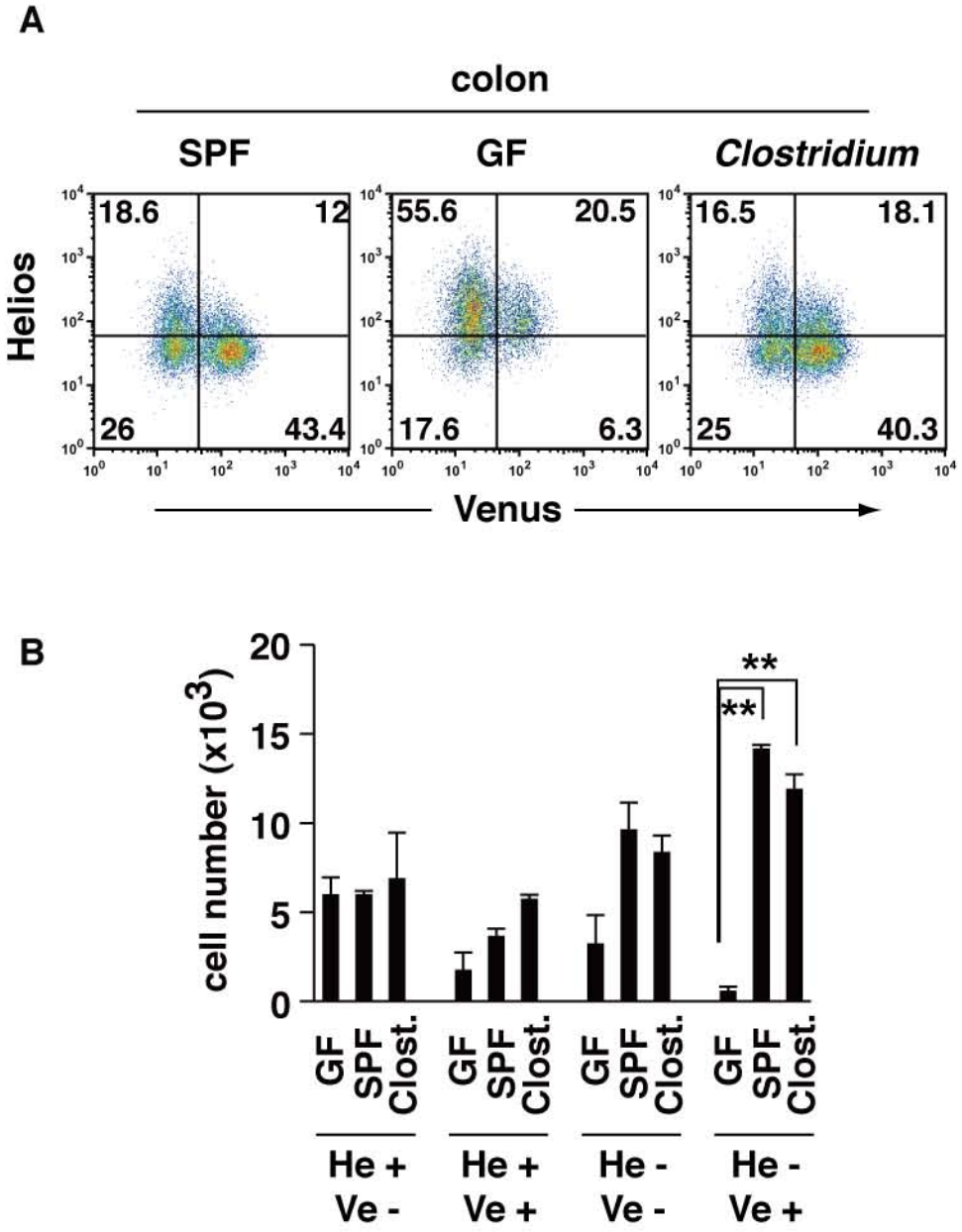

fig. S16 *Clostridium* induces the accumulation of CTLA4^{high}IL-10^{+} Helios^{-} subset of Treg cells. Germ-free (GF) IL10^{Venus} mice were colonized with 46 strains of *Clostridium* (Clost.). Lymphocytes from the colonic LP of SPF, GF, or Clost-associated mice were analyzed for the expression of CD4, Foxp3 and Helios by flow cytometry. Representative plots for Helios and Foxp3 expression gated on CD4^{+} cells are shown in (A), and absolute cell number of each subset (Ve, Venus; He, Helios) is shown in (B). Error bars indicate the SD (n =4 mice per group). $^{**}P < 0.001$ vs. GF, unpaired *t*-test.

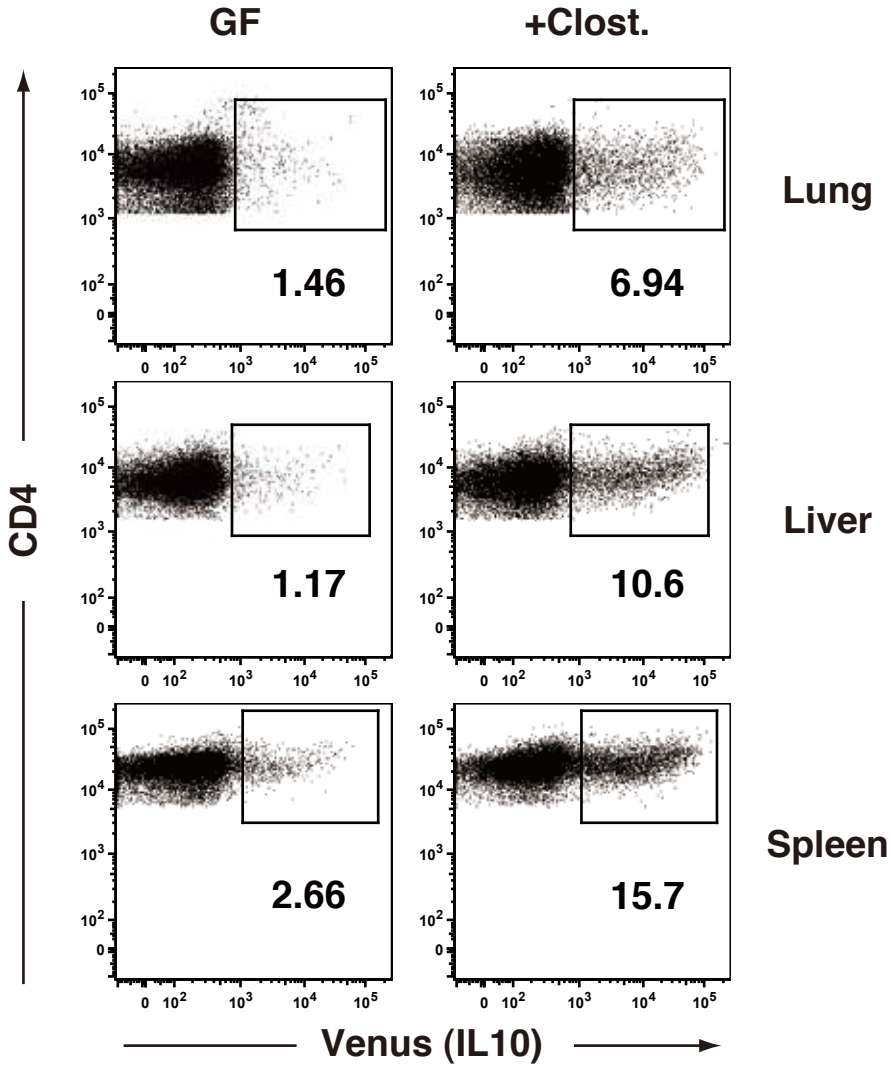

fig. S17 The appearance of IL10-expressing CD4$^+$ cells in lung, liver and spleen after colonization of *Clostridium*.

Germ-free (GF) IL10^{Venus} mice were orally inoculated with 46 strains of *Clostridium* (+Clost.). Three weeks after inoculation, lymphocytes were collected from lung, liver and spleen of GF or *Clostridium*-colonized mice were analyzed for Venus expression by CD4$^+$ cells. Representative plots from one of three independent experiments are shown. Numbers indicate the percentages of Venus$^+$ cells within the CD4$^+$ cell population.

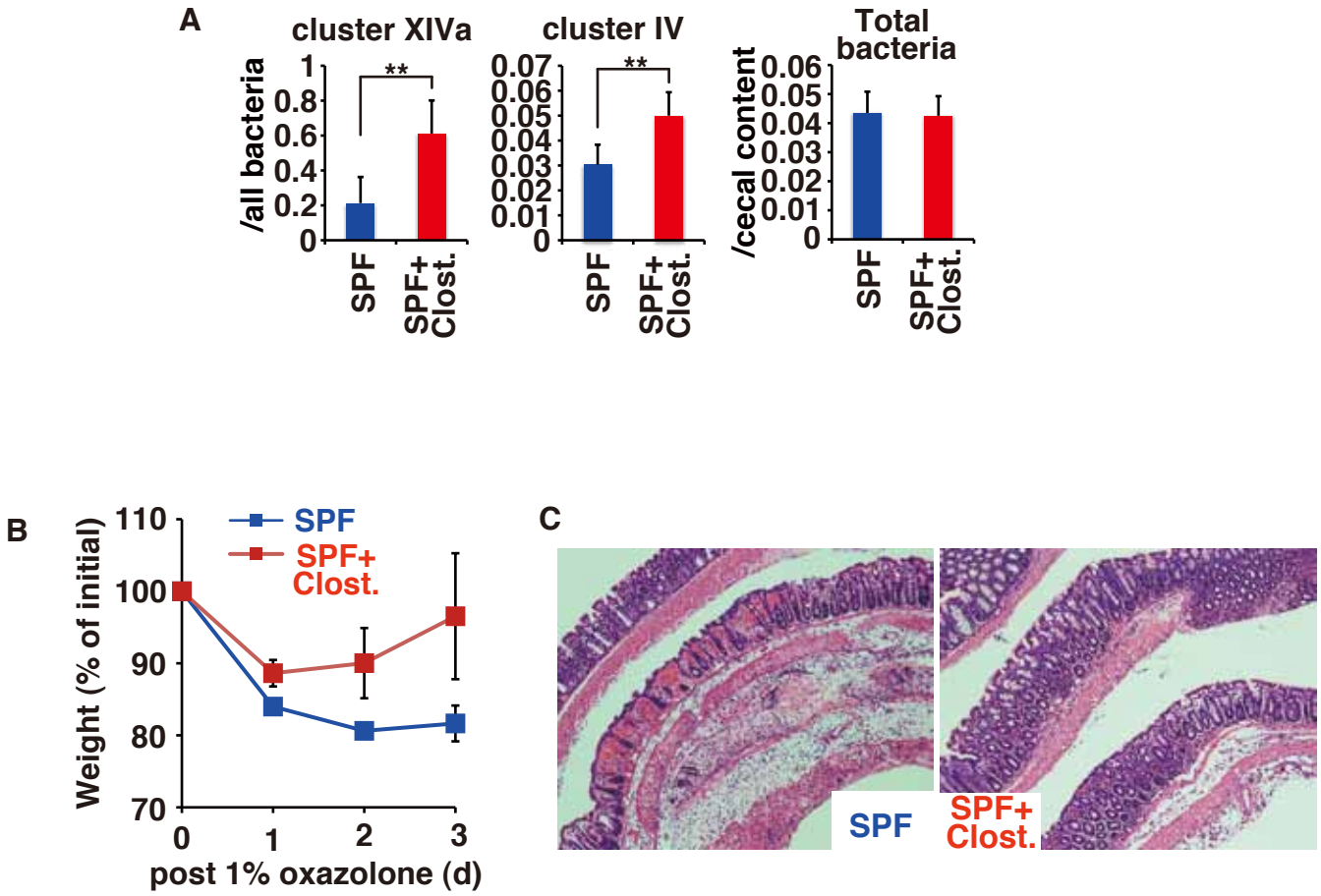

fig. S18

(A) Oral inoculation of *Clostridium* into 2-week old neonatal SPF mice resulted in *Clostridium* abundant mice. Quantitative PCR analysis of *Clostridium* cluster IV and XIVa groups and total bacterial 16S rRNA genes in the feces of 8-week old mice kept under SPF conditions after oral inoculation of 46 strains of *Clostridium* at 2 weeks old (SPF+Clost.). The feces from individual mice (n =5 mice per group) were analyzed and error bars indicate the SD.$^{**}P < 0.001$, unpaired *t*-test. The experiment was repeated more than three times with similar results.

(B, C) *Clostridium* abundabt mice showed mild symptoms of oxazolone-induced colitis. Untreated control (SPF) and *Clostridium*-abundant mice (SPF+Clost.) were pre-sensitized by epicutaneous application of 3% oxazolone followed by intra-rectal treatment with 1% oxazolone in 50% ethanol. Percent body weight of mice were shown in (B). Representative histology of colon tissue from control or *Clostridium*-abundant mice treated with oxazolone were shown in (C). Data are representative of two independent experiments. Error bars indicate the SD (n =4 mice per group).

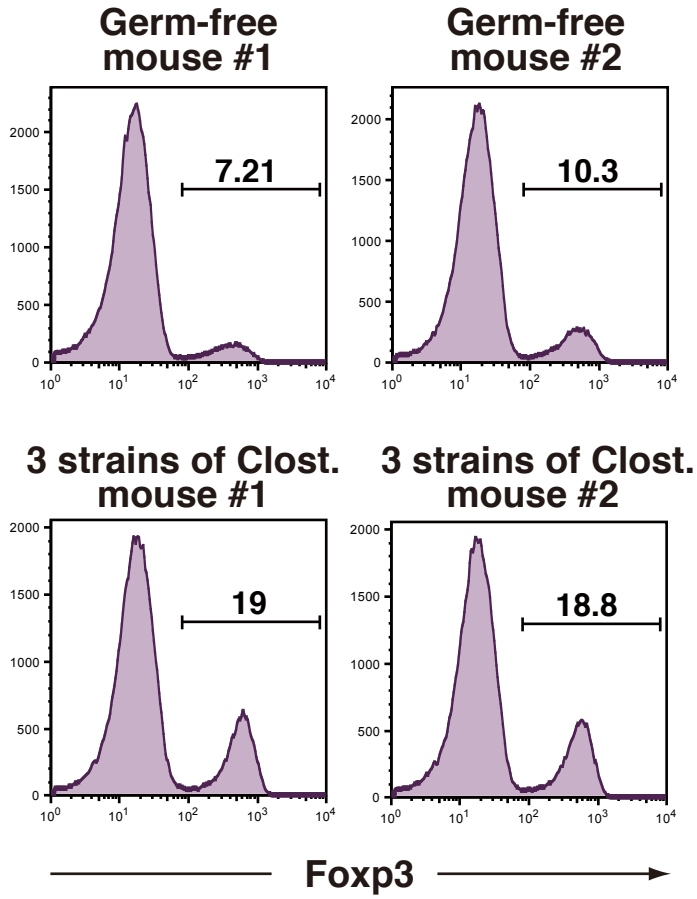

fig. S19 Treg cell induction by three strains of *Clostridium*.
GF Balb/c mice were colonized with three strains of *Clostridium* belonging to cluster IV (strains 22, 23 and 32 listed in fig.S8). Three weeks later, colonic Foxp3$^+$ Treg cells were analyzed by FACS. Representative histograms for Foxp3 expression gated on CD4$^+$ cells were shown.

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